UTILITY PATENT APPLICATION TRANSMITTAL LETTER (Only for new nonprovisional applications under 37 CFR 1.53(b))	Docket No. P04962US/UA/mw

To the Assistant Commissioner for Patents:

Transmitted herewith for filing is the patent application of:
Antoni BANAS, Line SANDAGER, Ulf STAHL, Anders DAHLQVIST, Marit LENMAN, Hans RONNE and Sten STYMNE
corresponding to Provisional application No. 99 850 169.6, filed November 12, 1999,
entitled: USE OF CLASS ENZYMES AND THEIR ENCODING GENES TO INCREASE THE OIL CONTENT IN TRANSGENIC ORGANISMS

Enclosed are:

X	22 pages of specification.
X	2 sheets of formal drawings.
	a newly-executed declaration of the inventor.
	a copy of an executed declaration of the inventor from prior application Serial No. , filed .
	incorporation by reference. The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied as indicated in the preceding box, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
	an assignment of the invention to , including assignment cover sheet.
	Information Disclosure Statement with Form PTO-1449.
	copies of the Information Disclosure Statement citations.
X	preliminary amendment.
X	return receipt postcard (MPEP 503), specifically itemized.
	applicant claims small entity status under 37 CFR 1.27.
	a certified copy of the Priority Document.
X	other: Data Entry Sheet. This application is filed without a declaration in order to preserve Convention priority.

If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information.*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP)

of prior application No. , filed .

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UTILITY PATENT APPLICATION TRANSMITTAL LETTER (continued)	Docket No. P04962US/UA/mw

CLAIMS AS FILED

	NO. FILED	NO. EXTRA	RATE	FEE
BASIC FEE			$ 710	$ 710
TOTAL CLAIMS	15 - 20 =	0	x$ 18	
INDEPENDENT CLAIMS	2 - 3 =	0	x$ 80	
MULTIPLE DEPENDENT CLAIM PRESENT			$ 270	
			TOTAL	$ 710
If applicant claims small entity status under 37 CFR 1.27, then divide total fee by 2, and enter amount here.			**SMALL ENTITY TOTAL**	$

X	A check in the amount of $710 to cover the filing fee is enclosed.
X	The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to Deposit Account No. 25-0120 in the name of Young & Thompson, as described below. A duplicate copy of this sheet is enclosed.
	Charge the amount of $ as filing fee.
X	Credit any overpayment.
X	Charge any additional fee required under 37 CFR 1.16 and 1.17, during the pendency of this application.
	Charge the issue fee set in 37 CFR 1.18 at the mailing of the Notice of Allowance.

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November 13, 2000

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APPLICATION INFORMATION

Title Line One:: USE OF A CLASS OF ENZYMES AND THEIR
Title Line Two:: ENCODING GENES TO INCREASE THE OIL
Title Line Three:: CONTENT IN TRANSGENIC ORGANISM
Total Drawing Sheets:: TWO
Formal Drawings?:: Yes
Application Type:: UTILITY
Docket Number:: P04962US/UA/mw

REPRESENTATIVE INFORMATION

Representative Customer Number:: 000466

CONTINUITY INFORMATION

This application is a::	CLAIMED PRIORITY OF
>Application One::	99 850 169.6
Filing Date::	NOVEMBER 12, 1999

PRIOR FOREIGN APPLICATION

Foreign Application One::	60/164,859
Filing Date::	NOVEMBER 12, 1999
Country::	SWEDEN
Priority Claimed::	Yes

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Antoni BANAS et al.

Serial No. (unknown)

Filed herewith

USE OF A CLASS OF ENZYMES AND THEIR ENCODING GENES TO INCREASE THE OIL CONTENT IN TRANSGENIC ORGANISMS

Box Non-fee Amendment

GROUP

Examiner

PRELIMINARY AMENDMENT

Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to the first Official Action and calculation of the filing fee, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Page 1, before line 1, insert the following paragraph:

--This application claims the benefit of U.S. Provisional Application No. 60/164,859, filed November 12, 1999." should appear as the first sentence of the description.--

IN THE CLAIMS:

Claim 3, line 1, change "claims 1 or 2" to --claim 1--.

Claim 4, line 1, change "claims 1, 2 or 3" to --claim 1--.

Claim 5, line 2, cancel "2, 3 or 4,".

Claim 10, line 1, change "claims 5-9" to --claim 5--.

Claim 12, line 1, change "any of the claims 5-9" to --claim 5--.

Claim 13, line 1, change "claims 11 or 12" to --claim 11--.

Claim 14, line 1, cancel "12 or 13".

Respectfully submitted,

YOUNG & THOMPSON

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November 13, 2000

Applicant : Scandinavian Biotechnology Research AB

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Title: USE OF A CLASS OF ENZYMES AND THEIR ENCODING GENES TO INCREASE THE OIL CONTENT IN TRANSGENIC ORGANISMS

FIELD OF THE INVENTION

The present invention relates to the use of a novel enzyme and its encoding gene for transformation. More specifically, the invention relates to the use of a gene encoding an enzyme with acyl-CoA : diacylglycerol acyltransferase activity. This gene expressed alone in transgenic organisms will increase the total amount of oil (*i.e.* triacylglycerols) that is produced.

BACKGROUND OF THE INVENTION

In oil crops like rape, sunflower, oilpalm etc., the oil (*i.e.* triacylglycerols) is the most valuable product of the seeds or fruits and other compounds such as starch, protein and fiber is regarded as by-products with less value. Enhancing the quantity of oil per weight basis at the expense of other compounds in oil crops would therefore increase the value of the crop. If enzymes regulating the allocation of reduced carbon into the production of oil can be upregulated by overexpression, the cells will accumulate more oil at the expense of other products. This approach could not only be used to increase the oil content in already high oil producing organisms such as oil crops, they could also lead to significant oil production in moderate or low oil containing crops such as soy, oat, maize, potato, sugar beats, and turnips as well as in microorganisms.

Development in genetic engineering technologies combined with greater understanding of the biosynthesis of triacylglycerols now makes it possible to transfer genes coding for key enzymes involved in the synthesis of triacylglycerols from a wild plant species or organisms of other kingdoms into domesticated oilseed crops. In this way, triacylglycerols can be produced in high purity and quantities at moderate costs.

It is known that the biosynthesis of triacylglycerols (TAG) in fat-accumulating tissues in animals (Bell & Coleman, 1983) and plants (Cao & Huang, 1986, Martin & Wilson 1983) as well as the accumulation of oil in microbial organisms such as bacteria (Ekundayo & Packter, 1994), yeast and other fungi (Ratledge 1989) can be catalyzed by acyl-CoA : diacylglycerol acyltransferases (DAGATs), enzymes that transfer an acyl-group from acyl-CoA to diacylglycerol, thus forming TAG.

During the past few years genes coding for DAGATs, have been identified in animals (Cases et al., 1998), plants (Hobbs et al., 1999; Lardizabal et al., 2000) and in microbes (Lardizabal et al., 1999). These DAGATs belong to two unrelated protein families.

The first type of DAGAT that was characterized, DAGAT A, has so far been found only in animals (Cases et al., 1998) and plants (Hobbs et al., 1999). These genes show sequence similarities to genes encoding acyl-CoA : cholesterol acyltransferase (ACAT). The mouse DAGAT A has 20 % amino acid sequence identity to the mouse ACAT (Cases et al., 1998). However, DAGATs A from plants and animals are more similar to each other than to ACAT. Thus, the mouse DAGAT A has 38 % amino acid sequence identity to the *Arabidopsis thaliana* DAGAT A (Hobbs et al., 1999). It is also approximately 80% identical to the human ACAT like protein ARGP1, which was suggested to be involved in TAG synthesis (Oelkers et al., 1998), indicating that ARGP1 is a DAGAT A.

The yeast *S. cerevisiae* contain 2 genes with sequence similarity to ACAT, *ARE1* and *ARE2* (Yang et al., 1996). The encoded proteins have approximately 24 % overall amino acid sequence identity to the mouse ACAT and 15% identity to the DAGAT A from mouse. It should be noted that they are both more similar to each other (45% amino acid sequence identity) than to either ACATs or DAGATs from higher eukaryotes. It is not possible to classify them as putative ACATs or DAGATs based on sequence similarities alone, since their evolutionary distances from both groups of higher eukaryotic enzymes are similar. However, experiments have shown that both Are1 and Are2 are ACATs, which together are responsible for all of the sterol ester synthesis that occurs in yeast (Yang et al., 1996; Yu et al., 1996). The possible involvement of Are1 and Are2 in the synthesis of TAG has also been studied (Yang et al., 1996; Yu et al., 1996). From these studies, it was concluded that Are1 and Are2 are not involved in TAG synthesis. Thus, there is no prior art to show that Are1 is a TAG synthesizing enzyme, nor can it be concluded, on the basis of homologies to ACAT like sequences already published, that Are1 is a DAGAT (Lassner and Ruczinsky, 1999).

The second family of DAGAT enzymes, the DAGAT B family, is unrelated to any other known proteins. These enzymes show no sequence homology to the mouse and plant ACAT like DAGAT A proteins (Lardizabal et al., 2000) or to any other known proteins.

DAGAT A and B are not the only enzymes that contribute to TAG biosynthesis. TAG can also be synthesized by an acyl-CoA independent reaction. Thus, the newly discovered enzyme phospholipid : diacylglycerol acyltransferase (PDAT) catalyses the formation of TAG by transferring an acyl group from the sn-2 position of a phospholipid to DAG (Dahlqvist et al., 1999; Ståhl, 1999).

SUMMARY OF INVENTION

This invention describes the identification of a gene encoding an enzyme that is partly responsible for TAG accumulation in yeast.

In a first embodiment, this invention is directed to the TAG synthesising enzyme comprising an amino acid sequence as set forth in SEQ ID NO 2 or a functional fragment, derivative, variant, ortologue or isoenzyme thereof.

The present invention further includes the nucleotide sequence as set forth in SEQ ID NO 1, as well as portions of the genomic sequence, the cDNA sequence, allelic variants, synthetic variants and mutants thereof. This includes sequences that codes for variants of the polypeptide set forth in the sequence listing including biologically active triacylglycerol synthesising enzymes as well as sequences that are to be used as probes, vectors for transformation or cloning intermediates.

Another aspect of the present invention relates to those polypeptides, which have at least 60% identity to SEQ ID NO 2. Preferred embodiments are polynucleotides that encode polypeptides with diacylglycerol acyltransferase activity.

In a different aspect, this invention relates to the use of these nucleotide sequences in recombinant DNA constructs to direct the transcription and translation of the diacylglycerol acyltransferase sequence of the present invention in a host organism or progeny thereof, including oil seeds, yeast and other fungi, as well as other oil accumulating organisms. Cells and organisms containing the diacylglycerol acyltransferase as a result of the production of the acyltransferase encoding sequence are also included within the scope of the invention.

Of particular interest is the expression of the nucleotide sequences of the present invention from transcription initiation regions that are preferentially expressed in plant seed tissues. It is contemplated that the gene sequence may be synthesized, especially when there is interest to provide plant-preferred codons.

In a different embodiment, this invention also relates to methods of using a DNA sequence encoding a said protein of the present invention for increasing the oil-content within the cells of different organisms.

Further, the invention makes possible a process for the production of triacylglycerol, which comprises growing transgenic cells or organisms under conditions whereby any of the nucleotide sequences discussed above are expressed in order to produce an enzyme in these cells with the ability to transfer a fatty acid from acyl-CoA to diacylglycerol, thus forming triacylglycerol.

Moreover, triacylglycerols produced by the aforementioned process are included in the scope of the present invention.

The present invention can be essentially characterized by the following aspects:

1. Use of a nucleic acid sequence encoding an enzyme catalysing the transfer of a fatty acid from acyl-CoA to diacylglycerol for the production of triacylglycerol (TAG) by genetic transformation of an oil-producing organism with said sequence in order to be expressed in this organism and result in an active enzyme in order to increase the oil content of the organism.
The nucleic acid sequence is derived from the sequence shown in SEQ ID NO. 1, from the *Saccharomyces cerevisiae ARE1* gene (genomic clone or cDNA), or from a nucleic acid sequence or cDNA that contain nucleotide sequences coding for a protein with an amino acid sequence that is 60% or more identical to the amino acid sequence as presented in SEQ. ID. NO. 2.

2. Transgenic organisms comprising, in their genome or on a plasmid, a nucleic acid sequence according to the above, transferred by recombinant DNA technology. The transgenic organisms are selected from the group consisting of fungi, plants and animals. Preferably the transgenic organisms agricultural plants and preferably said nucleotide sequence is expressed under the control of a storage organ specific promoter. Alternatively, the nucleotide sequence is expressed under the control of a seed-specific promoter.

3. Oils from organisms according to aspect 2.

4. A protein encoded by a DNA molecule according to SEQ ID NO. 1 or a functional (enzymatically active) fragment thereof. Alternatively, the protein produced in an organism as specified in aspect 2, which has the amino acid sequence set forth in

SEQ ID NO. 2 or an amino acid sequence with at least 60 % homology to said amino acid sequence. Preferably the protein is isolated from *Saccharomyces cerevisiae.*

5. Use of a protein as specified in aspect 4 in the production of triacylglycerols.

6. Triacylglycerols according aspect 5.

DETAILED DESCRIPTION OF THE INVENTION

The invention now having been generally described will be more readily understood by reference to the following drawings and examples, which are included for the purpose of illustration only, and are not intended to limit scope of the present invention.

Description of the figures:

Figure 1. *In vitro* DAGAT activity in a yeast strain (SCY62) that overexpresses the *ARE1* gene. Aliquots of microsomal membranes prepared from the control strain (lane A) or the *ARE1* overexpressing strain (lane B) were assayed for DAGAT activity according to Method A described in Material and Methods. The radioactive triacylglycerol synthesised was visualised and quantified as cpm (figures in brackets) on the TLC plate by electronic autoradiography (Instant Imager, Packard, US). Abbreviations used in the figure: triacylglycerol (TAG) and unesterified fatty acids (FA).

Figure 2. *In vitro* DAGAT activity in a PDAT DAGAT B double mutant, a PDAT DAGAT B *ARE1* triple mutant, and in the same triple mutant containing a plasmid that overexpresses the *ARE1* gene.
The radioactive triacylglycerols (TAG) synthesised in microsomes from the double mutant, H1226 (lane A), the triple mutant, H1236 (lane B) and the same triple mutant containing a plasmid that overexpresses the *ARE1* gene (lane C) were visualised on a TLC plate by electronic autoradiography (Instant Imager, Packard, US).

BRIEF DESCRIPTION OF THE SEQ ID:

SEQ ID NO. 1: Genomic DNA sequence of the *Saccharomyces cerevisiae ARE1* gene , ORF YCR048W.

SEQ ID NO. 2: The amino acid sequence of the open reading frame YCR048W from *Saccharomyces cerevisiae.*

EXAMPLES

EXAMPLE 1 - **Triacylglycerol accumulation is reduced in yeast cells that lack the *ARE1* gene**

Materials and Methods

Yeast strains. Yeast strains used in this study were congenic to the W303-1A (Thomas & Rothstein, 1989) background. An *are1* mutant strain, H1111, with the genotype *MATα are1-Δ::HIS3 ADE2 can 1-100 leu2-3,112 trp1-1 ura3-1*, was generated by crossing the two strains SCY60 *(MATα are1-Δ::HIS3 ade2-1 can 1-100 leu2-3,112 trp1-1 ura3-1*) and SCY61 *(MAT***a** *are2-Δ::LEU2 ADE2 can 1-100 his3-11,15 trp1-1 ura3-1*) (Yang et al., 1996) and dissecting tetrads. As a wild type control, we used SCY62 *(MAT***a** *ADE2 can 1-100 his3-11,15 leu2-3 trp1-1 ura3-1)* (Yang et al., 1996). Yeast mutant strains disrupted in YNR008w and YOR245c encoding yeast DAGAT B and PDAT, respectively, and the *ARE1* gene were constructed through a series of yeast transformations using the lithium acetate method. Linear DNA fragments used for the disruption of the YOR245c and YNR008w genes were created as follows. Primers specific for YOR245c (300 bases upstream, CAGCATTGACGTAATGGGAA, and downstream, AAAGCCAAAAAGAGAAGGACA, of the gene) were constructed and the gene was synthesised using PCR from SCY62 genomic DNA. The PCR-fragment was blunt-ended and ligated into pUC119 previously cleaved with the restriction enzyme *Sma*I. The resulting plasmid, YOR245c-pUC119, was then digested with *ClaI/Stu*I and dephosphorylated to prevent religation. The marker KanMX4 was obtained by digestion of the plasmid pFA6a by *SmaI/SacI*. The blunted KanMX4 fragment was then ligated into the YOR245c-pUC119 vector between the *Cla*I and *Stu*I sites within the YOR245c open reading frame. A linear fragment containing the resulting YOR245c::KanMX4 disruption cassette was finally obtained through cleavage by *Bam*HI/*Nde*I. The linear fragment used to disrupt the YNR008w gene was constructed in a similar manner as the YOR245c::KanMX4 fragment. The YNR008w gene was amplified from SCY62 genomic DNA, cloned into the pBluescript vector (Dahlqvist et al., 2000) and digested with restriction enzyme *BbsI/Mun*I. The *TRP1* marker was then ligated between the *Bbs*I and *Mun*I sites in the YNR008w-pBluescript plasmid, and a linear fragment containing the disruption cassette was obtained by *Bam*HI/*Pst*I digestion. The single PDAT mutant, H1079, with the genotype MATa *pdat-Δ::TRP1 ADE2 leu2-3,112 ura3-1 his3-11,15 trp1-1*,

was generated by transforming the wild type strain SCY62 with the linear YNR008w::TRP1 fragment. The PDAT DAGAT B double mutant, H1226, with the genotype MATa *pdat-Δ::TRP1 dagat B-Δ::KanMX4 ADE2 leu2-3,112 ura3-1 his3-11,15 trp1-1*, was constructed in an identical manner by transforming H1079 with the linear YOR245c::KanMX4 fragment. An *ARE1* PDAT double mutant, H1224, with the genotype MATα *are1-Δ::HIS3 pdat-Δ::TRP1 ADE2 can 1-100 leu2-3,112 ura3-1 trp1-1*, was generated by transforming H1111 with the linear YNR008w::TRP1 fragment. The triple mutant strain, H1236, with the genotype MATα *are1-Δ::HIS3 pdat-Δ::TRP1 dagat B-Δ::KanMX4 ADE2 leu2-3,112 ura3-1 trp1-1*, was constructed by transforming H1224 with the linear YOR245c::KanMX4 fragment.

Yeast Cultivations. Yeast cells were cultivated at 28 or 30°C on a rotary shaker in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose). Transformed cells were grown in synthetic medium (Sherman et al., 1986) lacking uracil and supplemented with 2 % (vol/vol) glycerol and 2% (vol/vol) ethanol.

Lipid Analysis. The lipid content of the yeast cells was determined as described by Dahlqvist et al. (2000) and is presented as nmol of fatty acid (FA) per mg dry weight yeast.

Results

The lipid content of a mutant yeast strain (SCY60), in which the *ARE1* gene was disrupted, was analyzed and compared to wild type yeast cells (SCY62) at different stages of growth. In *are1* mutant cells, harvested in exponential phase after 10 hours of cultivation, the total amount of lipid, measured as nmol FA per dry weight yeast, was not significantly different from the wild type yeast (table 1), nor did the amount of fatty acids accumulated into TAG differ strongly between the wild-type and the *are1* mutant. The effect of the *are1* disruption on oil accumulation in stationary phase cells was analysed in an experiment were the yeast cells were pre-cultivated for 24 h in liquid YPD medium. The cells were then harvested and re-suspended in minimal medium (Meesters et al, 1996), supplemented with 16 g/l glycerol, to the original volume of the growth culture. In this glycerol supplemented minimal medium the yeast cells will enter stationary phase under conditions suitable for TAG accumulation. After further cultivation for 24 h, the cells were harvested and their lipid composition was determined. The total lipid content in the *are1* mutant was 15% less than in the wild type. The TAG amount in the *are1* mutant was almost 40 % lower than in the wild type, whereas the polar lipid content did not differ significantly between the *are1* mutant and the wild type yeast (table 1).

Two other genes, YNR008w and YOR254c (Ståhl, 1999; Dahlqvist, et al., 2000; Lardizabal et al., 2000) have recently been shown to be involved in TAG synthesis in yeast. These genes encode a PDAT and a DAGAT B protein, respectively. A yeast strain disrupted in all three genes (ARE1, YNR008w and YOR254c) and a yeast strain with disruptions in only the PDAT and DAGAT B genes were made and they are here named the triple and double mutant, respectively. The TAG content of the double mutant was 48 % of the wild type (table 2), whereas the amount of TAG accumulated in the triple mutant was only 4% of the level in the wild type yeast. By comparing the amounts of TAG accumulated in the double and triple mutants it is clear that Are1 protein contributes to TAG synthesis in yeast.

In summary, these experiment clearly show that the product of the *ARE1* gene contributes to TAG accumulation in yeast.

Table 1. ***Lipid content in ARE1 mutant (SCY60) and wild type (SCY62) yeast cells.*** The lipid accumulation in yeast disrupted in the *ARE1* gene (*are1* mutant) was analysed at different stages of growth and compared to the control wild type yeast. The lipid composition of cells in exponential growth was analysed after 10 hours of cultivation in YPD medium at 28 °C. Yeast cells in stationary phase was prepared by pre-cultivating the cells on liquid YPD medium for 24 hours at 28 °C, after which the cells were harvested, re-suspended in minimal medium (Meesters et al, 1996) supplemented with 16 g/l glycerol, and cultivated for an additional 24 hours at 28 °C. The content of sterol esters, TAG, other neutral lipids, and polar lipids was determined as nmol fatty acids (FA) per mg of dry yeast weight.

	SCY62 (nmol FA / mg)		SCY60 (nmol FA / mg)	
	10h	**48h**	**10h**	**48h**
Sterol esters	15	24	12	19
Triacylglycerol	6	44	8	28
Other neutral lipids	4	6	4	5
Polar lipids	65	74	63	74
Total lipids	90	148	87	126

Table 2. ***Lipid content in the PDAT DAGATB double mutant strain (H1226), in the PDAT DAGATB ARE1 triple mutant strain (H1236) and in wild type yeast cells (SCY62).*** The different yeast strains, all of which contained the empty expression plasmid pJN92 (Ronne et al., 1991), were cultivated in YNB medium to which 2 % (v/v) of galactose was added at an A_{600} of 4. The cells were harvested after an additional 22 hours growth and the content of sterol esters, TAG, other neutral lipids, and polar lipids was determined as nmol fatty acids (FA) per mg of dry yeast weight.

	SCY62 (nmol FA / mg)	**H1226** (nmol FA / mg)	**H1236** (nmol FA / mg)
Sterol esters	13	10	1
Triacylglycerol	163	78	7
Other neutral lipids	17	16	41
Polar lipids	58	66	44
Total lipids	251	170	87

EXAMPLE 2 - **Triacylglycerol accumulation is increased in yeast cells that overexpress the *ARE1* gene.**

Material and Methods

For induced overexpression of the *ARE1* gene, a 2001 bp *EheI/Ecl*136II fragment from the plasmid YEP 3-16 (Yang et al., 1996) was cloned into the *Bam*HI site of the *GAL1* expression vector pJN92 (Ronne et al., 1991), thus generating pUS5. The wild type yeast strain SCY62 *(MATa ADE2 can 1-100 his3-11,15 leu2-3 trp1-1 ura3-1)* (Yang et al., 1996), was transformed with the pUS5 and cultivated at 28 °C on a rotary shaker in synthetic medium (Sherman et al., 1986) lacking uracil and supplemented with 2 % (vol/vol) glycerol and 2 % (vol/vol) ethanol. The *GAL1* promoter was induced after 43 h of growth by the addition of 2 % (wt/vol) final concentration of galactose. Cells were harvested after an additional 24 hours of growth. Wild type (SCY62) cells transformed with the empty vector, pJN92, and cultivated under identical conditions were used as a control. The lipid content of the yeast cells was determined as described by Dahlqvist et al. (2000) and is presented as nmol of fatty acid (FA) per mg dry weight yeast.

Results

The effect of overexpression of the *ARE1* gene on lipid accumulation was studied by transforming the wild-type yeast (strain SCY62) with a plasmid containing the *ARE1* gene under control of the galactose-induced *GAL1* promotor (Table 3). Overexpression of the *ARE1* gene from this promoter had no strong effect on the growth rate as determined by optical density measurements. However, the total lipid content in yeast cells that overexpressed *ARE1* was 1.4 fold higher than in the control yeast transformed with an empty expression vector (Table 3). The elevated lipid content in yeast cells overexpressing *ARE1* is mostly due to a 50% increase in the TAG content, but the amount of sterol esters also increased significantly in these cells, as compared to the control. These results clearly demonstrate that the gene product of *ARE1*, in addition to its earlier reported involvement in the synthesis of sterol esters (Yang et al., 1996), also is involved in TAG synthesis. The elevated levels of TAG achieved in the *ARE1* overexpressing cells also clearly demonstrate the potential use of the *ARE1* gene in increasing the oil content in transgenic organisms.

Table 3. ***Lipid content in yeast cells that overexpress the ARE1 gene.*** Yeast cells (SCY 62) transformed with the *ARE1* gene under the control of the *GAL1* promotor in the pJN92 vector were cultivated as described in the Material and Method section. Yeast cells (SCY62), transformed with an empty vector, cultivated under identical conditions were used as control. The cells were harvested and the content of sterol esters, triacylglycerols, other neutral lipids and polar lipids was determined as nmol fatty acids (FA) per mg dry yeast weight.

	SCY62 (nmol FA / mg)	**SCY62 overexpressing *ARE1*** (nmol FA / mg)
Sterol esters	19	27
Triacylglycerol	160	239
Other neutral lipids	30	32
Polar lipids	48	56
Total lipids	257	354

EXAMPLE 3 - The ARE1 gene product has diacylglycerol acyltransferase activity.

Materials and Methods

In vitro diacylglycerol acyltransferase (DAGAT) activity was analyzed, in microsomal fractions prepared from yeast cells, by using one of the following methods.

Method A: A wild type yeast (strain SCY62) was transformed with a plasmid (pUS5) containing the *ARE1* gene under the control of a *GAL1* promotor (described in Material and Methods in Example 2). The transformed yeast was cultivated at 28°C in defined YNB medium lacking uracil. The expression of the *ARE1* gene was induced by the addition of 2 % (v/v) galactose after 8 hours growth and the cells were harvested after an additional 17 hours. Microsomal membranes were prepared from the transformed yeast by resuspending 1g of yeast (fresh weight) in 8 ml of ice-cold buffer (20 mM Tris-Cl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5 % (v/v) glycerol, 1 mM DTT, 0.3 M ammonium sulphate) in a 12 ml glass tube to which 4 ml of glass beads (diameter 0.45 – 0.5 mm) were added. The glass tube was heavily shaken (3 x 60 s) with a MSK cell homogenizer (B. Braun Melsungen AG, Germany). The suspension was centrifuged at 20 000 g for 15 min at 6 °C and the resulting supernatant was centrifuged at 100 000g for 2 h at 6 °C. The resulting pellet, containing microsomal membranes, was resuspended in 0.1 M K-phosphate (pH 7.2) buffer and stored at –80 °C. DAGAT activity was analyzed in aliquots of microsomal membranes (50 µl), corresponding to 10 nmol phosphatidylcholine, to which 1 µmol of dioleoyl-PG and 0.25 µmol of dioleoyl-DAG emulsified in 50 µl of buffer containing 190 mM HEPES-NaOH, pH 7.5, 125 mM $MgCl_2$, 30 mM CHAPS, 2.5 mg/ml BSA and 2 nmol [^{14}C]-palmitoyl-CoA (2775 dpm/nmol), were added. The reaction mixture was incubated at 30°C for 30 min. The lipids were then extracted in chloroform and separated using thin layer chromatography on silica gel 60 plates in hexane / diethyl ether / acetic acid (80:20:1). The radioactive lipids were visualized and quantified on the plates by electronic autoradiography (Instant Imager, Packard, US).

Method B: The PDAT DAGAT B double mutant (H1226) and the PDAT DAGAT B *ARE1* triple mutant (H1236), described in Material and Methods in Example 1, were transformed with the empty expression plasmid (pJN92). A transformant expressing the *ARE1* gene under the control of the *GAL1* promotor was generated by transforming the triple mutant H1236 with the plasmid pUS5 (described in Material and Methods in Example 2). All yeast transformants were cultivated in YNB medium to which 2 % (v/v) of galactose was added at an A_{600} of 4. The cells were harvested after an additional 6 hours growth and microsomes

were prepared using a modification of the procedure of Dahlqvist et al. (2000). Yeast cells (0.2 g) were resuspended in 1.5 ml of ice-cold buffer (20 mM Tris·Cl pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5 % (vol/vol) glycerol, 1 mM DTT, 0.3 M ammonium sulfate) in a 2 ml Eppendorf tube containing 0.2 ml glass beads (0.45-0.5 mm in diameter). The tube was heavily shaken (3 x 60 s) in a cell homogenizer (Mini Bead Beater). The homogenized yeast was centrifuged at 1350 x g for 20 min at 4 °C, and the resulting supernatant was subsequently centrifuged at 150 000 x g for 1 h at 4 °C. The pellet was re-suspended in 0.1 M potassium phosphate (pH 7.2), and stored at –80 °C. Dihexanoyl-DAG (5 nmol) dissolved in chloroform was added to micro tubes and the chloroform was evaporated under a stream of N_2. Aliquots (90 µl) of microsomal fractions corresponding to 150 µg protein, in a buffer consisting of 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$, and 1 mg/ml BSA were added to the tubes and the suspension was thoroughly mixed. Finally, 10 µl of [^{14}C]-palmitoyl-CoA (20 nmol, 5000 dpm/nmol) was added, and the mixtures were incubated at 30 °C for 15 min. Lipids were extracted from the reaction mixture into chloroform (Bligh & Dyer, 1959) and separated by TLC on silica gel 60 plates (Merck). The TLC plate was first developed in chloroform / methanol / acetic acid / water (85:15:10:3.5) for 80 mm. The dried plate was then developed in hexane / diethyl ether / acetic acid (80:20:1.5) for 180 mm. The radioactive lipids were visualized and quantified on the plates by electronic autoradiography (Instant Imager, Packard).

Results

Microsomal membranes prepared from the transformed yeast overexpressing the *ARE1* gene and from control yeast transformed with an empty plasmid (pJN92) were assayed for DAGAT activity according to Method A in Materials and Methods. The amount of radiolabelled TAG synthesized from [^{14}C]palmitoyl-CoA in microsomal membranes prepared from the *ARE1* overexpressor was increased with 66 % as compared to the control yeast (Fig 1). DAGAT activity was also assayed in microsomal membranes prepared from the PDAT DAGAT B double mutant strain (H1226) and the PDAT DAGAT B *ARE1* triple mutant strain (H1236) cells (Method B). In the double mutant, with a functional *ARE1* gene, TAG with two hexanoyl and one [^{14}C]palmitoyl chain, was synthesized from added dihexanoyl-DAG and [^{14}C]palmitoyl-CoA. This synthesis was barely detectable in the triple mutant (figure 2) where the *ARE1* gene was disrupted. However, the in vitro synthesis of TAG was restored in triple mutant cells transformed with a plasmid expressing the *ARE1* gene. This clearly shows that the *in vitro* synthesis of TAG in these yeast mutants correlates with the presence of a

functional *ARE1* gene and that the protein encoded by the *ARE1* gene possesses DAGAT activity.

EXAMPLE 4 -Triacylglycerol accumulation is increased in the seeds of *Arabidopsis thaliana* that express the *ARE1* gene.

Material and methods

The *ARE1* gene was amplified from the yeast genome using the proof reading enzyme polymerase pfu (Promega). An *Eco*R1 and *Xba*1 restriction enzyme site was introduced respectively into the 5' and 3' ends of this fragment to allow directional cloning of the fragment. The PCR fragment was cloned into the vector pBluescript (Stratagene). The insert derived from this plasmid was then cloned downstream of a napin promoter fragment (Stålberg *et al.*, 1993) in the vector pPGTV-KAN (Becker *et al.*, 1993). This plasmid was transformed into *Agrobacterium* strain GV3301. Transformed *Agrobacterium* cells were then used to transform root explants from *Arabidopsis thaliana* (Valvekens *et al.*, 1992). The lipid content in *Arabidopsis* seeds was determined by methylation of fatty acids. Fatty acids in the oil of proximately 2-3 mg of seeds were methylated in 2 ml 2 % (vol/vol) H_2SO_4 in dry methanol for 90 min at 90 °C. The fatty acid methyl esters were extracted with hexane and analyzed by GLC through a 50 m × 0.32 mm CP-Wax58-CB fused-silica column (chrompack), methylheptadecanoic acid was used as internal standard.

Results

A. thaliana was transformed with the *ARE1* gene under the control of a napin promoter, which is seed specific and active during the major phase of oil accumulation. The oil content was analyzed in seeds from single T2 plants derived from four independent transformation events (Table 4). The results showed that in three lines between 50 % and 100 % of the T2 plants generated seeds with statistically significant elevated oil content as compared to the oil content in the seeds from the control plants. The oil content was elevated with up to 18 % in the seeds expressing *ARE1*. One line (28-1) had the same oil content as the seeds from the control plants.

Table 4. ***Accumulation of oil in seeds from Arabidopsis thaliana transformed with the ARE1 gene.***

T2 plants transformed with the *ARE1* gene under the control of the napin promotor and control plants transformed with an empty vector were cultivated in a growth chamber under controlled conditions. The oil content in mature seeds of these plants was determined by GLC analyses and is presented as nmol fatty acids (FA) per mg seed.

		Transformants			
	control	**28-1**	**28-2**	**28-3**	**28-4**
Number of T2 plants analyzed	4	6	2	6	11
Number of T2 plants with significant increased seed oil content*	-	0	2	3	9
nmol FA per mg seed in T2 plant with highest oil content	1535±114	1562±28	1753±53	1641±82	1818±18

* Calculated with the mean difference two-sided test at $\alpha = 5$ and based on the average oil content of 4 control plants.

SEQUENCE LISTING

1 GENERAL INFORMATION

i) APPLICANT: Scandinavian Biotechnology Research AB

ii)TITLE OF INVENTION: Use of a class of enzymes and their encoding genes to increase oil content in transgenic organisms

iii) Number of sequences: 2

2) INFORMATION FOR SEQ ID NO:1:

i) SEQUENCE CHARACTERISTICS:
A) LENGTH: 1833 bases
B) TYPE: nucleic acid
C) STRANDEDNESS: single
D) TOPOLOGY: linear

ii) MOLECULE TYPE: DNA

iii) SEQUENCE DESCRIPTION:: SEQ ID NO: 1:

ATGACGGAGA CTAAGGATTT GTTGCAAGAC GAAGAGTTTC TTAAGATCCG 50

CAGACTCAAT TCCGCAGAAG CCAACAAACG GCATTCGGTC ACGTACGATA 100

ACGTGATCCT GCCACAGGAG TCCATGGAGG TTTCGCCACG GTCGTCTACC 150

ACGTCGCTGG TGGAGCCAGT GGAGTCGACT GAAGGAGTGG AGTCGACTGA 200

GGCGGAACGT GTGGCAGGGA AGCAGGAGCA GGAGGAGGAG TACCCTGTGG 250

ACGCCCACAT GCAAAAGTAC CTTTCACACC TGAAGAGCAA GTCTCGGTCG 300

AGGTTCCACC GAAAGGATGC TAGCAAGTAT GTGTCGTTTT TTGGGGACGT 350

GAGTTTTGAT CCTCGCCCCA CGCTCCTGGA CAGCGCCATC AACGTGCCCT 400

TCCAGACGAC TTTCAAAGGT CCGGTGCTGG AGAAACAGCT CAAAAATTTA 450

CAGTTGACAA AGACCAAGAC CAAGGCCACG GTGAAGACTA CGGTGAAGAC 500

TACGGAGAAA ACGGACAAGG CAGATGCCCC CCCAGGAGAA AAACTGGAGT 550

CGAACTTTTC AGGGATCTAC GTGTTCGCAT GGATGTTCTT GGGCTGGATA 600

GCCATCAGGT GCTGCACAGA TTACTATGCG TCGTACGGCA GTGCATGGAA 650

TAAGCTGGAA ATCGTGCAGT ACATGACAAC GGACTTGTTC ACGATCGCAA 700

TGTTGGACTT GGCAATGTTC CTGTGCACTT TCTTCGTGGT TTTCGTGCAC 750

TGGCTGGTGA AAAAGCGGAT CATCAACTGG AAGTGGACTG GGTTCGTTGC 800

AGTGAGCATC TTCGAGTTGG CTTTCATCCC CGTGACGTTC CCCATTTACG 850

TCTACTACTT TGATTTCAAC TGGGTCACGA GAATCTTCCT GTTCCTGCAC 900

TCCGTGGTGT TTGTTATGAA GAGCCACTCG TTTGCCTTTT ACAACGGGTA 950

TCTTTGGGAC ATAAAGCAGG AACTCGAGTA CTCTTCCAAA CAGTTGCAAA 1000

AATACAAGGA ATCTTTGTCC CCAGAGACCC GCGAGATTCT GCAAAAAAGT 1050

TGCGACTTTT GCCTTTTCGA ATTGAACTAC CAGACCAAGG ATAACGACTT 1100

CCCCAACAAC ATCAGTTGCA GCAATTTCTT CATGTTCTGT TTGTTCCCCG 1150

TCCTCGTGTA CCAGATCAAC TACCCAAGAA CGTCGCGCAT CAGATGGAGG 1200

TATGTGTTGG AGAAGGTGTG CGCCATCATT GGCACCATCT TCCTCATGAT 1250

GGTCACGGCA CAGTTCTTCA TGCACCCGGT GGCCATGCGC TGTATCCAGT 1300

TCCACAACAC GCCCACCTTC GGCGGCTGGA TCCCCGCCAC GCAAGAGTGG 1350

TTCCACCTGC TCTTCGACAT GATTCCGGGC TTCACTGTTC TGTACATGCT 1400

CACGTTTTAC ATGATATGGG ACGCTTTATT GAATTGCGTG GCGGAGTTGA 1450

CCAGGTTTGC GGACAGATAT TTCTACGGCG ACTGGTGGAA TTGCGTTTCG 1500

TTTGAAGAGT TTAGCAGAAT CTGGAACGTC CCCGTTCACA AATTTTTACT 1550

AAGACACGTG TACCACAGCT CCATGGGCGC ATTGCATTTG AGCAAGAGCC 1600

AAGCTACATT ATTTACTTTT TTCTTGAGTG CCGTGTTCCA CGAAATGGCC 1650

ATGTTCGCCA TTTTCAGAAG GGTTAGAGGA TATCTGTTCA TGTTCCAACT 1700

GTCGCAGTTT GTGTGGACTG CTTTGAGCAA CACCAAGTTT CTACGGGCAA 1750

GACCGCAGTT GTCCAACGTT GTCTTTTCGT TTGGTGTCTG TTCAGGGCCC 1800

AGTATCATTA TGACGTTGTA CCTGACCTTA TGA 1833

2) INFORMATION FOR SEQ ID NO:2:

i) SEQUENCE CHARACTERISTICS:

A) LENGTH: 610 amino acids

B) TYPE: amino acid

D) TOPOLOGY: linear

ii) MOLECULE TYPE: protein

iii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Thr Glu Thr Lys Asp Leu Leu Gln Asp Glu Glu Phe Leu Lys Ile
 1               5                  10                  15

Arg Arg Leu Asn Ser Ala Glu Ala Asn Lys Arg His Ser Val Thr Tyr
            20                  25                  30

Asp Asn Val Ile Leu Pro Gln Glu Ser Met Glu Val Ser Pro Arg Ser
        35                  40                  45

Ser Thr Thr Ser Leu Val Glu Pro Val Glu Ser Thr Glu Gly Val Glu
    50                  55                  60

Ser Thr Glu Ala Glu Arg Val Ala Gly Lys Gln Glu Gln Glu Glu Glu
65                  70                  75                  80

Tyr Pro Val Asp Ala His Met Gln Lys Tyr Leu Ser His Leu Lys Ser
                85                  90                  95

Lys Ser Arg Ser Arg Phe His Arg Lys Asp Ala Ser Lys Tyr Val Ser
            100                 105                 110

Phe Phe Gly Asp Val Ser Phe Asp Pro Arg Pro Thr Leu Leu Asp Ser
        115                 120                 125

Ala Ile Asn Val Pro Phe Gln Thr Thr Phe Lys Gly Pro Val Leu Glu
    130                 135                 140

Lys Gln Leu Lys Asn Leu Gln Leu Thr Lys Thr Lys Thr Lys Ala Thr
145                 150                 155                 160

Val Lys Thr Thr Val Lys Thr Thr Glu Lys Thr Asp Lys Ala Asp Ala
                165                 170                 175

Pro Pro Gly Glu Lys Leu Glu Ser Asn Phe Ser Gly Ile Tyr Val Phe
            180                 185                 190

Ala Trp Met Phe Leu Gly Trp Ile Ala Ile Arg Cys Cys Thr Asp Tyr
        195                 200                 205
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Tyr Ala Ser Tyr Gly Ser Ala Trp Asn Lys Leu Glu Ile Val Gln Tyr
    210                 215                 220

Met Thr Thr Asp Leu Phe Thr Ile Ala Met Leu Asp Leu Ala Met Phe
225                 230                 235                     240

Leu Cys Thr Phe Phe Val Val Phe Val His Trp Leu Val Lys Lys Arg
                245                 250                 255

Ile Ile Asn Trp Lys Trp Thr Gly Phe Val Ala Val Ser Ile Phe Glu
            260                 265                 270

Leu Ala Phe Ile Pro Val Thr Phe Pro Ile Tyr Val Tyr Tyr Phe Asp
        275                 280                 285

Phe Asn Trp Val Thr Arg Ile Phe Leu Phe Leu His Ser Val Val Phe
    290                 295                 300

Val Met Lys Ser His Ser Phe Ala Phe Tyr Asn Gly Tyr Leu Trp Asp
305                 310                 315                     320

Ile Lys Gln Glu Leu Glu Tyr Ser Ser Lys Gln Leu Gln Lys Tyr Lys
                325                 330                 335

Glu Ser Leu Ser Pro Glu Thr Arg Glu Ile Leu Gln Lys Ser Cys Asp
            340                 345                 350

Phe Cys Leu Phe Glu Leu Asn Tyr Gln Thr Lys Asp Asn Asp Phe Pro
        355                 360                 365

Asn Asn Ile Ser Cys Ser Asn Phe Phe Met Phe Cys Leu Phe Pro Val
    370                 375                 380

Leu Val Tyr Gln Ile Asn Tyr Pro Arg Thr Ser Arg Ile Arg Trp Arg
385                 390                 395                     400

Tyr Val Leu Glu Lys Val Cys Ala Ile Ile Gly Thr Ile Phe Leu Met
                405                 410                 415

Met Val Thr Ala Gln Phe Phe Met His Pro Val Ala Met Arg Cys Ile
            420                 425                 430

Gln Phe His Asn Thr Pro Thr Phe Gly Gly Trp Ile Pro Ala Thr Gln
        435                 440                 445

Glu Trp Phe His Leu Leu Phe Asp Met Ile Pro Gly Phe Thr Val Leu
    450                 455                 460

Tyr Met Leu Thr Phe Tyr Met Ile Trp Asp Ala Leu Leu Asn Cys Val
465                 470                 475                     480

Ala Glu Leu Thr Arg Phe Ala Asp Arg Tyr Phe Tyr Gly Asp Trp Trp
                485                 490                 495

Asn Cys Val Ser Phe Glu Glu Phe Ser Arg Ile Trp Asn Val Pro Val
            500                 505                 510

His Lys Phe Leu Leu Arg His Val Tyr His Ser Ser Met Gly Ala Leu
        515                 520                 525
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His Leu Ser Lys Ser Gln Ala Thr Leu Phe Thr Phe Phe Leu Ser Ala
    530                 535                 540

Val Phe His Glu Met Ala Met Phe Ala Ile Phe Arg Arg Val Arg Gly
545                 550                 555                 560

Tyr Leu Phe Met Phe Gln Leu Ser Gln Phe Val Trp Thr Ala Leu Ser
                565                 570                 575

Asn Thr Lys Phe Leu Arg Ala Arg Pro Gln Leu Ser Asn Val Val Phe
            580                 585                 590

Ser Phe Gly Val Cys Ser Gly Pro Ser Ile Ile Met Thr Leu Tyr Leu
        595                 600                 605

Thr Leu
    610
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CLAIMS

1. Use of a nucleic acid sequence encoding an enzyme catalysing the transfer of a fatty acid from acyl-CoA to diacylglycerol for the production of triacylglycerol (TAG) by genetic transformation of an oil-producing organism with said sequence in order to be expressed in this organism and result in an active enzyme in order to increase the oil content of the organism.

2. Use according to claim 1, wherein said nucleic acid sequence is derived from the sequence shown in SEQ ID NO. 1.

3. Use according to claims 1 or 2, wherein said sequence is derived from the *Saccharomyces cerevisiae ARE1* gene (genomic clone or cDNA).

4. Use according to claims 1, 2 or 3, wherein a nucleic acid sequence or cDNA is used that contain nucleotide sequences coding for a protein with an amino acid sequence that is 60% or more identical to the amino acid sequence as presented in SEQ. ID. NO. 2.

5. Transgenic organisms comprising, in their genome or on a plasmid, a nucleic acid sequence according to claim 1, 2, 3 or 4, transferred by recombinant DNA technology.

6. Transgenic organisms according to claim 5, which are selected from the group consisting of fungi, plants and animals.

7. Transgenic organisms according to claim 6, which are selected from the group of agricultural plants.

8. Transgenic organisms according to claim 7 which are selected from the group of agricultural plants and where said nucleotide sequence is expressed under the control of a storage organ specific promoter.

9. Transgenic organisms according to claim 8 which are selected from the group of agricultural plants and where said nucleotide sequence is expressed under the control of a seed-specific promoter.

10. Oils from organisms according to claims 5-9.

11. A protein encoded by a DNA molecule according to SEQ ID NO. 1 or a functional (enzymatically active) fragment thereof.

12. A protein produced in an organism as specified in any of the claims 5-9, which has the amino acid sequence set forth in SEQ ID NO. 2 or an amino acid sequence with at least 60 % homology to said amino acid sequence.

13. A protein as specified in claims 11 or 12 that is isolated from *Saccharomyces cerevisiae*.

14. Use of a protein as specified in claim 11, 12 or 13 in the production of triacylglycerols.

15. Triacylglycerols according to claim 14.

ABSTRACT

The present invention relates to the use of a novel enzyme and its encoding gene for transformation. More specifically, the invention relates to the use of a gene encoding an enzyme with acyl-CoA : diacylglycerol acyltransferase activity. This gene expressed alone in transgenic organisms will increase the total amount of oil (*i.e.* triacylglycerols) that is produced.

FIGURE 1/2

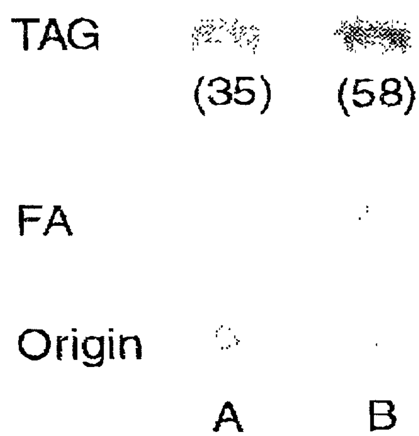

FIGURE 2/2